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Proteomic characterization of sperm radial spokes identifies a novel spoke protein with an ubiquitin domain

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ABSTRACT

Radial spokes are T-shaped protein complexes important for the regulation of axonemal dyneins in eukaryotic cilia and flagella. Using a functional proteomics approach, we identified six spoke proteins in sperm flagella of the ascidian *Ciona intestinalis*. Many of the domain/motif structures in spoke proteins are commonly found in flagella of both *Ciona* sperm and *Chlamydomonas*, but interestingly they often distribute over non-orthologous protein components. A novel 116 kDa protein named CMUB116 has both an ubiquitin domain and an IQ motif. It has orthologs in vertebrates, but not in *Chlamydomonas*. Furthermore, the results obtained by immunological analysis provide strong indication that CMUB116 is located at the stalk of radial spokes, where it is associated with MORN40.

Structured summary:
MINT-7148244: *CMUB116* (genbank_protein_gi:BAH59277) and *MORN40* (genbank_protein_gi:BAH59284) *colocalize* (MI:0403) by *cosedimentation* (MI:0027)
MINT-7148179: *Ci-RSP3* (uniprotkb:Q8T898) *physically interacts* (MI:0915) with *tubulin alpha* (uniprotkb:Q8MVT7), *LRR37* (uniprotkb:Q8T896), *CMUB116* (genbank_protein_gi:BAH59277), *Ci-SRP4/6* (genbank_protein_gi:BAH59283), *AK58* (genbank_protein_gi:BAM59278), *tubulin beta* (genbank_protein_gi:XP_002130315), *NDK/DPY26* (genbank_protein_gi:BAH59279), *MORN40* (genbank_protein_gi:BAH59284), *ARM37* (genbank_protein_gi:BAH59280), *NDK/DPY26* (genbank_protein_gi:NP_001161489), *LC8* (genbank_protein_gi:BAH59282) and *Ci-RSP9* (genbank_protein_gi:NP_001154962) by *anti bait coimmunoprecipitation* (MI:0006)
MINT-7148272: *Ci-RSP3* (uniprotkb:Q8T898) and *MORN40* (genbank_protein_gi:BAH59284) *colocalize* (MI:0403) by *cosedimentation* (MI:0027)

1. Introduction

The axonemes in eukaryotic flagella and cilia are sophisticated micromachinery generally constructed by nine outer doublet microtubules and two central pair singlet microtubules. Their motility is produced by active sliding of outer doublet microtubules by axonemal dyneins [1]. Several lines of evidence indicate that radial spokes and the central pair play an essential role in the regulation of dynein arms, with the central pair determining the flagellar bending plane by sending signals through radial spokes to dynein arms [2–4]. *Chlamydomonas* mutants lacking radial spoke or central pair apparatus have paralyzed flagella [5,6]. Several extragenic suppressor mutations, however, allow these paralyzed flagella to be motile by changing the phosphorylation states of dynein heavy chains or dynein regulatory complex [7–11]. Protein kinase inhibitors mimic this bypass effect, implying that radial spokes play important roles in the regulation of dynein activity through protein phosphorylation [12,13].

Isolation of apparently intact radial spokes by 0.5 M KI treatment of *Chlamydomonas* axonemes resulted in the identification of 23 different polypeptides by two-dimensional gel [14], including a 102 kDa component [15]. These radial spoke proteins (RSPs) are termed as RSP1 to RSP23. By a proteomic approach, Yang and her colleagues characterized 18 RSPs in *Chlamydomonas* [16]. Despite these characterizations in *Chlamydomonas*, RPSs in metazoan sperm flagella have been only partially characterized.

Abbreviations: MALDI-TOF/MS, matrix assisted laser desorption/ionization-time of flight mass spectrometry; RSP, radial spoke protein; LRR, leucine-rich repeat; NDK, nucleoside diphosphate kinase; MORN, membrane occupation and recognition nexus; ARM, armadillo repeat; LC, light chain

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We use *Ciona intestinalis*, a primitive chordate with a simple body plan, for the molecular characterization of metazoan axonemes [17–20]. In an initial study, we cloned a cDNA showing homology to *Chlamydomonas* RSP3 and isolated a complex containing RSP3 and the leucine-rich protein LRR37 [21]. This was followed by the identification of the radial spoke proteins Ci-RSP4/6, Ci-MORN40, Ci-AxHsp40 and Ci-Rsp33 (Ci-RSP9) in axonemal KI extracts [20]. Immunoprecipitation using an anti-Ci-RSP3 antibody yielded distinct 16 specific bands. In this study, we identified additional six radial spoke proteins in the immunoprecipitates by a proteomic approach. By comparing radial spoke proteins from *Chlamydomonas* and *Ciona*, we show that the domain/motif structures are distributed over different components but are all derived from the radial spoke complex. Furthermore, we characterized a novel radial spoke protein that has both a calmodulin binding IQ motif and an ubiquitin domain, which we refer to as CMUB116 (116 kDa protein with both calmodulin and ubiquitin domains).

2. Materials and methods

2.1. Experimental materials

Adults of *C. intestinalis* were supplied by the Education and Research Center of Marine Bio-Resources, Tohoku University, and Department Zoology, Graduate School of Science, Kyoto University (National Bioresource Project, NBRP). They were kept under constant light for several days until sperm were properly accumulated in sperm duct.

2.2. Extraction of radial spoke

Sperm flagella and axonemes are prepared from *C. intestinalis* sperm pellet as described previously [20]. High salt extraction with a solution containing 0.6 M KCl, 20 mM Tris–HCl, pH 8.0, 1 mM $MgSO_4$, 0.5 mM EGTA, and 0.2 mM dithiothreitol was repeated twice for careful removal of outer arm dyneins [20]. Then KCl-extracted axonemes were treated with a buffer containing 0.5 M KI as reported previously [20]. After centrifugation at 100 000×*g* for 40 min at 4 °C, the clear supernatant was collected and dialyzed against excess amount of buffer A (0.15 M KCl, 2 mM $MgCl_2$, 1 mM EGTA, 20 mM Tris–HCl, 0.5 mM DTT, pH 8.0) for 6 h with twice changes of the buffer. The extract in the dialysis bag was centrifuged at 12 000×*g* for 15 min at 4 °C and the supernatant was used for immunoprecipitation of radial spokes with anti-Ci-RSP3 antibody.

2.3. Separation of radial spoke components

To isolate a protein complex containing CMUB116, the high salt extract of the axonemes was applied on 5–20% sucrose density gradient made with the buffer containing 0.15 M KCl, 2 mM $MgCl_2$, 0.5 mM EGTA, 10 mM Tris–HCl, pH 8.0, 0.2 mM DTT), and centrifuged in a HITACHI Himac CP65β with P28S2 rotor at 26 000 rpm for 21 h. The fractions of 500 μl each were collected. The main fractions showing immunoreactivity to both anti-CMUB116 and -MORN40 antibodies were pooled and subjected to MonoQ anion exchange column (Amersham Pharmacia Biotech) with an AKTA fast-performance liquid chromatography (FPLC) system (Amersham Pharmacia Biotech). The column was equilibrated with 0.15 M KCl, 2 mM $MgCl_2$, 1 mM EGTA, 20 mM Tris–HCl, 0.5 mM DTT, pH 8.0, and the proteins were eluted at a flow rate of 1.0 ml/min by a gradient of KCl up to 2 M. Fractions of 0.25 μl each were collected.

2.4. Antibodies and immunohistochemistry

Polyclonal antibodies raised in mouse were used in this study. Antibodies against RSP3 and LRR37 were prepared as described in [21] and those against AxHsp40, RSP9 (RSp33) and MORN40 were described in [20]. In the present study, three antibodies (anti-CMUB116, anti-NDK/DPY26 and anti-Ci-Spag6) were newly prepared. For the construction of a protein expression vector, *Ciona* cDNA clone cits36b15, ciad045o15, or cits026b10 was picked up as a representative clone for CMUB116, NDK/DPY26 or Ci-Spag6, respectively. The open reading frames in the cDNA inserts were amplified by polymerase chain reaction. Primers used were 5′-CGCGGAATTCATGGCCGACGAACAACCA-3′ (sense) and 5′-CGCGG TCGACATTGTTACATTAAGTGGG-3′ (antisense) for CMUB116, 5′-CGCGGAATTCATGTCACCTATTCTAATG-3′ (sense) and 5′-CGCG GTCGACATACAAATACACTCTCTG-3′ (antisense) for NDK/DPY26, 5′-CGCGGGATCCAGGAAGAATGTCGCCACT-3′ (sense) and 5′-CGC GGAATTCTGTTTACCACCCCACCAG-3′ (antisense) for Ci-Spag6. The amplified cDNA and pET 32a (+) were cleaved with proper combination of restriction enzymes, ligated each other after removal of small DNA fragments by S-400 spin column (Amersham Biosciences, Tokyo), and transformed into the host cell AD494. Protein expression was induced by the addition of 2 mM isopropyl-1-thio-β-D-galactopyranoside. Recombinant proteins were extracted from bacteria by 5 mM imidazole, 0.5 M NaCl, 20 mM Tris–HCl, pH 8.0, 6 M urea, and 0.5% Triton X-100 and purified by Ni^{2+}-immobilized His-Bond metal chelation resin (Novagen, Madison, WI). Proteins were dialyzed against phosphate-buffered saline and used as antigen to raise antibodies. Polyclonal antibodies against the recombinant proteins were raised in Balb/c mice. The antigen was emulsified with Freund's complete adjuvant, and mice were given three subcutaneous injections at intervals of 10 d. Immunofluorescent microscopy and immunogold electron microscopy were performed according to Satouh et al. [20]. For immunogold labeling, the primary antibody was incubated with KCl-extracted axonemes, which were prepared by the treatment of the axonemes in advance with 0.6 M KCl, 20 mM Tris–HCl, pH 8.0, 1 mM $MgSO_4$, 0.5 mM EGTA, 0.2 mM dithiothreitol for 30 min on ice to remove outer arm dyneins as described in Section 2.2.

2.5. Immunoprecipitation

Antiserum was diluted to 1:20 with phosphate-buffered saline (PBS). Diluted antiserum (1 ml) was mixed with 100 μl of protein G-Sepharose beads (Amersham Biosciences). After gentle agitation for 2 h, the beads were washed with PBS repetitively. Antibody was then immobilized onto the beads with 2.5 mM disuccinimidyl suberate (Pierce Chemical, Rockford, IL) in PBS for 1 h. The beads were washed with 50 mM glycine, pH 2.5, four times to terminate cross-linking reaction and to ablate non-specific binding, followed by equilibration with buffer A in a spin column. Triton X-100 was added to KI extract to 1% and applied to the column equilibrated with buffer A containing 1% Triton X-100. The column was repetitively washed with excess amount of buffer A containing 1% Triton X-100. Proteins were eluted with 1 ml of 100 mM glycine (pH 2.5).

2.6. Peptide mass fingerprinting

Protein bands were excised from a polyacrylamide gel and in-gel digestion was performed according to Toda et al. [22]. Peptide mass spectra were obtained on an Autoflex II TOF/TOF in a reflector mode with the mass range from 1000 to 3000 Da (Bruker Daltonik GmbH, Germany). PerMS/MSCITS search was performed as described previously [17] with the molecular mass tolerance at 0.15 Da. A protein database of amino acid sequences, CIPRO, was

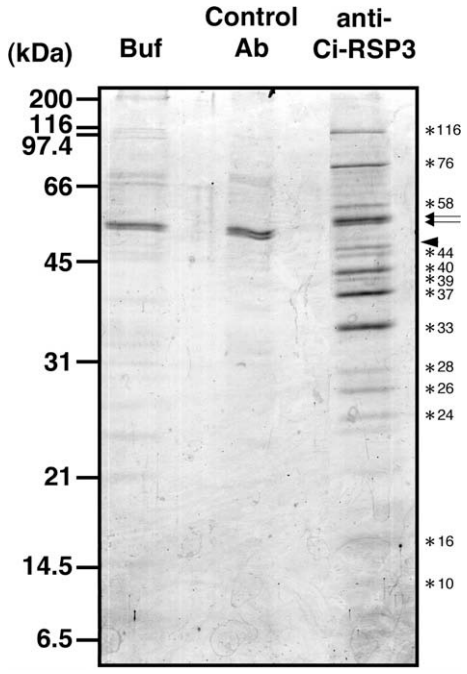

Fig. 1. Isolation of radial spoke proteins by immunoprecipitation. RSP3-associated proteins were eluted with 100 mM glycine (pH 2.5). As a result of comparison with the control eluate, 14 proteins were identified including Ci-RSP3. Proteins were separated by 12% polyacrylamide gel and were visualized by Coomassie Brilliant Blue. Proteins subjected to peptide mass fingerprinting are indicated by asterisks, along with their molecular masses on the right (in kilodalton). The arrows show the 53- and 52 kDa bands of α- and β-tubulins. The arrowhead indicates the 49 kDa band of Ci-RSP3.

constructed from a cDNA-based gene model kyotograil2005 (http://hoya.zool.kyoto-u.ac.jp/download.html), a predicted JGI gene model (http://genome.jgi-psf.org/Cioin2/Cioin2.download.ftp.html) and PROCITS [17]. The peptide mass spectra was searched against CIPRO database using MASCOT with the molecular mass tolerance at 0.3 Da. Oxidation of methionine residue was counted as a variable modification, while carboxymethylation of cystein residue was counted as a fixed modification. Proteins hit with a combined peptide score higher than 59 were considered significant.

2.7. Chemical cross-linking

Radial spoke proteins in *Ciona* 0.5 M KI extract was immunoprecipitated by anti-Ci-RSP3 antibody. The precipitate was eluted and dialyzed against the buffer containing 0.15 M KCl, 20 mM HEPES–NaOH, pH 8.0, 1 mM $MgSO_4$, and 0.5 mM EGTA. It was then chemically cross-linked with various final concentration of a zero-length cross-linker, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Sigma Chemical, St. Louis, MO). The reaction was terminated by adding the SDS buffer for SDS–PAGE. Proteins were separated by 8% SDS–PAGE gel and the cross-linked products were detected by Western blotting using anti-MORN40 antibody.

2.8. Computational analysis

Translation of DNA into amino acids, calculation of molecular mass, design of PCR primers, and estimation of isoelectric points were done by GENETYX-Mac software. BLASTP program against DDBJ/GenBank/EMBL database was used to search for homologous proteins. ProfileScan and SMART (Simple Modular Architecture Research Tool [23] (http://smart.embl-heidelberg.de) and was used for prediction of functional sites and for domain/motif search.

3. Results and discussion

The axonemal 0.5 M KI extract was immunoprecipitated by anti-Ci-RSP3 antibody. Comparison of protein bands with the control eluate, 16 specific bands were found in the immunoprecipitate (Fig. 1). It included seven proteins with the molecular mass of 76-, 53-, 52-, 49-, 40-, 37-, and 33 kDa, showing electrophoretic mobility identical to those in previously purified radial spoke subcomplex by column chromatographies [20]. Another nine proteins in the immunoprecipitate had the molecular weight of 116-, 58-, 44-, 39-, 28-, 26-, 24-, 16- and 10 kDa. These 16 protein bands were subjected to peptide mass fingerprinting by matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF/MS) (Tables 1 and 2). In addition to previously identified seven components including 52- and 53 kDa tubulins, seven proteins with the molecular weight of 116-, 58-, 44-, 37-, 33-, 26- and 10 kDa could be newly identified (Table 1). These proteins were named in accordance with molecular mass, domain/motif search and with homology to *Chlamydomonas* spoke proteins as CMUB116 (116 kDa), AK58 (58 kDa), NDK/DM44 (44 kDa), ARM37 (37 kDa), Ci-RSP9 (33 kDa; previously named as CiRSp33 [20]), NDK/DPY26

Table 1
Identification of *Ciona* radial spoke proteins by peptide mass fingerprinting.

Protein (kDa)	Protein ID	Score	Size (Da)	Name	Accession No.	Gene Discription by BLASTp Search
116	CLSTR_08511	170	88 396	CMUB116	AB501292	*Homo sapiens* cDNA FLJ35834 fis, clone TESTI2006615
76	CLSTR_09453	88	59 961	Ci-RSP4/6	AB501298	–
58	KYOTOGRAIL_261_2_1	119	55 135	AK58	AB501293	*Xenopus tropicalis* Hypothetical protein MGC76170
53	CLSTR_00246	101	50 754	Tubulin-α		–
52	CLSTR_00619	67	50 426	Tubulin-β		–
47	CLSTR_08279	153	37 454	Ci-RSP3	BAB85844	–
44	KYOTOGRAIL_69_21_1	76	43 966	NDK/DM44	AB501294	Mouse nucleoside diphosphate kinase 7 (nm23-M7)
40	CLSTR_02632	127	32 964	MORN40	AB501299	–
39	No significant Hit	–	–	–		–
37[a]	CLSTR_10996	96	34 958	LRR37	BAB85845	–
37[a]	KYOTOGRAIL_73_5_1	90	37 230	ARM37	AB501295	Mouse adult male testis cDNA clone:4933431K05
33	CLSTR_08605	94	31 267	Ci-RSP9[b]	AB501300	–
28	No significant Hit	–	–	–		–
26	CLSTR_08973	78	28 845	NDK/DPY26	AB501296	*Homo sapiens* nm23-H5 protein
24	No significant Hit	–	–	–		–
16	No significant Hit	–	–	–		–
10	PROCITS.01185.3.1.0	–	–	LC8	AB501297	*Ciona savignyi* dynein light chain protein

[a] Protein band with the molecular weight of 37 kDa was identified as a mixture of two proteins by MASCOT.
[b] This proteins was named CiRSp33 in the previous paper [20].

(26 kDa) and LC8 (10 kDa) (Fig. 2). The ARM37 was overlapped with Ci-AxHsp40 in a band on SDS–PAGE but they were clearly separated from each other by two-dimensional gel electrophoresis (data not shown). Three proteins with molecular masses of 28-, 24- and 16 kDa have not been identified in this study.

Among *Ciona* spoke proteins, Ci-RSP4/6, Ci-RSP3, LRR37, Ci-AxHsp40, ARM37, Ci-RSP9 or LC8 showed significant similarities to *Chlamydomonas* RSP4/6, RSP3, RSP15, RSP16, RSP14, RSP9 or RSP22, respectively. MORN40 showed sequence similarity to *Chlamydomonas* MORN repeat protein RSP10, RSP1 and FAP207, but the similarity was not found in the entire region of these homologs. NDK/DPY26 contained both NDK and Dpy-30 domains. The NDK domain showed high similarity to one of the human NDKs, nm23-H5, and to *Chlamydomonas* RSP23 (*E* value = 4e−45). RSP23 contains NDK domain but no Dpy-30 domain that is found in another spoke component RSP2 (Fig. 2 and Table 3). CMUB116, AK58 and NDK/DM44 could not be assigned to any known radial spoke protein of *Chlamydomonas*.

Table 2
Coverage and peptide fragment matched in Mascot search for each protein.

Protein	Coverage (%)	Peptide fragment matched		
CMUB116	35	TVEPEPNEPSVTEDATTER	VPASLLHFSYEGK	RKPFLGGFR
		EQLGSSDVITVRLIKENNEYEDVVVEIER	ISGVEYHHANAQTQAKTRPPSGTEK	
		DCQTVFER	YTTYQEYFAR	AVIVLQSYFR
		SLVRELK	EKEER	LEELGHINEELEGPER
		AALCALLEQETQLITSIGR	YTEMDTQYTIR	EHDCK
		EIIELIDR	GVVEANLEGLR	NPLFNPEAAR
		VPQDPSTLRK	KVQQLDNAAR	QDYSHYR
		HTLARIYFSR	KVTGSQRLPGPGEVR	
AK58	44	DATKKPLR	IAVIGPPASGK	LWVQMIEER
		RGFVLEGFPQNR	HLVVLDAPNTVLVER	EFVSGGERVPNSLVTK
		LLVPEGISETETRDRLNEYSRNIDGVLATYPHIHK		ITDPISGERYHTLYNPPR
		ADVLAQTYNFINTQLRSEAPHTPRIVLIGATGSGK		CLQNYK
		LMQLDASSRGWVLHGYPLTRDQAEGLFDAGLQPNRIYFFDIPDDCVIER		AQMVQMTR
NDK/DM44	30	RYQFLYYPKDSSIEMFDIK	LEDLYIGSK	QLTFVDFGDQFTESK
		TFALIKPDATSKLGVILNGLR	WLEMLGPSDPSEAR	AMFGTDELK
		ANAEEFYEIYK	QLCGPSDPEIGR	
ARM37	44	ISYTLPPNIDATK	ELNDDTLITR	ALMTLCDLVHDPEK
		LLSDADSTVR	STEVLYIMATHNVGR	LFDDNVDIVR
		ELILDTLHFCMR	SGVMPVLTELLDHSDPSVR	LLTDVDASVKSSAAGALMTIAITTK
		ALAPEGR	EDDPESAAVAR	
NDK/DPY26	47	TLGIVKPDAMDKVVEIEDIILRNGFTILQK	KVHLSPEQASDFYVEHYGK	SIASWRELIGPTNPFK
		ETHPGCLRDTYGKDQTRNAVHGSDSFHSAER	FMFHDSIVEPIAVGQATK	GLTQLCK
LC8	58	KAVIKNADMAEDMQQDAVDCATQALEKYNIEKDIAAYIKK		KYNPTWHCICGR

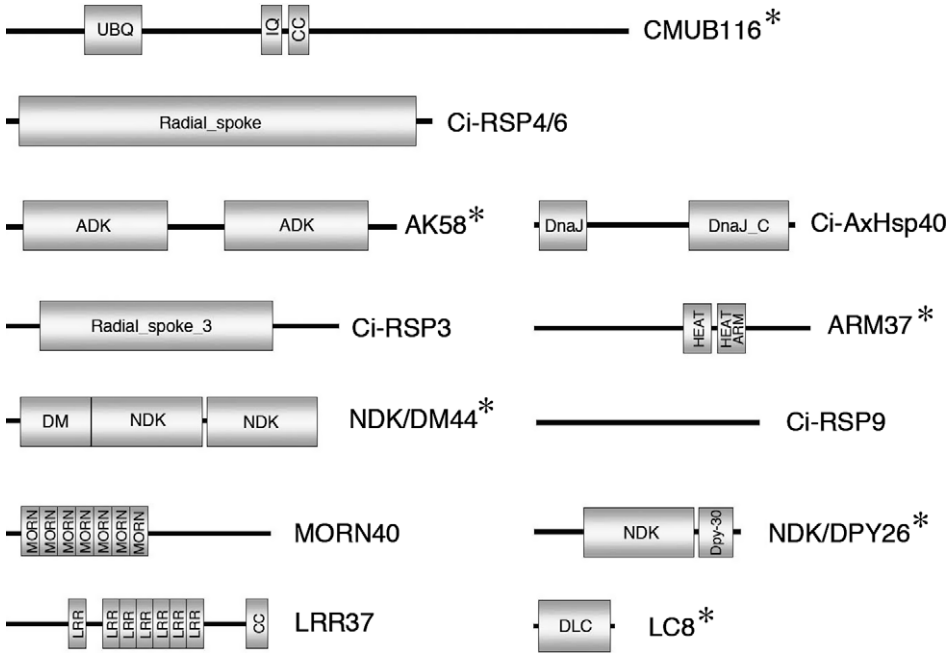

Fig. 2. Summary of domain/motif structures of *Ciona* radial spoke proteins. UBQ, ubiquitin domain; IQ, IQ calmodulin binding motif; CC, coiled coil region; ADK, adenylate kinase; DM, DM-10 domain; NDK, nucleoside diphosphate kinase; MORN, membrane occupation and recognition nexus repeat; LRR, leucine-rich repeat; DnaJ and DnaJ-C, DnaJ-J and DnaJ-C molecular chaperone homology domain; HEAT, Huntingtin, elongation factor 3, PR65/A subunit of protein phosphatase A, and the TOR; ARM, armadillo repeat; DPY, Dpy-30 motif. Proteins labeled with asterisks are newly identified in this study.

Table 3
Domain/motif structure of *Ciona* radial spoke proteins and their comparison with those in *Chlamydomonas*.

Ciona intestinalis					*Chlamydomonas reinhardtii*		
Protein	kDa	Domain/motif	BLASTP against CrDB	*E* value	Protein	kDa	Domain/motif
CMUB116	116	Ub, IQ	(FAP253)[a]	1e−55	RSP18	210	
Ci-RSP4/6	76		RSP4/RSP6	2e−42/5e−44	RSP19	140	
AK58	58	AK	Chlre3:194134[b]	3e−30	RSP17	124	GAF
Ci-RSP3	49	AKAP	RSP3	2e−57	RSP1	123	MORN
NDK/DM44	44	NDK, DM-10	(FAP67)	1e−102	RSP2	118	Dpy-30, GAF, 1-8-14
MORN40	40	MORN	RSP10/(FAP207)/RSP1	2e−25/1e−20/4e−20	RSP23	102	NDK, IQ
LRR37	39	LRR	RSP15 (?)[c]	2e−09	RSP13	98	
Ci-AxHsp40	37	DnaJ, DnaJ-C	RSP16	2e−72	RSP3	86	AKAP
ARM37	37	ARM	RSP14	4e−06	RSP4	76	
Ci-RSP9	33		RSP9	6e−22	RSP5	69	Aldo-keto reductase
28 kDa	28				RSP6	67	
NDK/DPY26	26	NDK, Dpy-30	RSP23	4e−45	RSP7	58	RIIa, EF
24 kDa	24				RSP14	41	ARM
16 kDa	16				RSP8	40	ARM
LC8	10	(LC8)	RSP22		RSP15	38	LRR
					RSP16	34	DnaJ, DnaJ-C
					RSP9	26	
					RSP10	24	MORN
					RSP11	22	RIIa
					RSP12	20	PPI
					RSP20	18	EF(CaM)
					RSP21	16	
					RSP22	8	(LC8)

The components are listed in the order of molecular mass.
[a] Protein showing high similarity to CMUB116 but has no ubiquitin domain.
[b] Protein ID assigned in *Chlamydomonas* genome database ver. 3.
[c] Only a short sequence is available for RSP15 in *Chlamydomonas* database

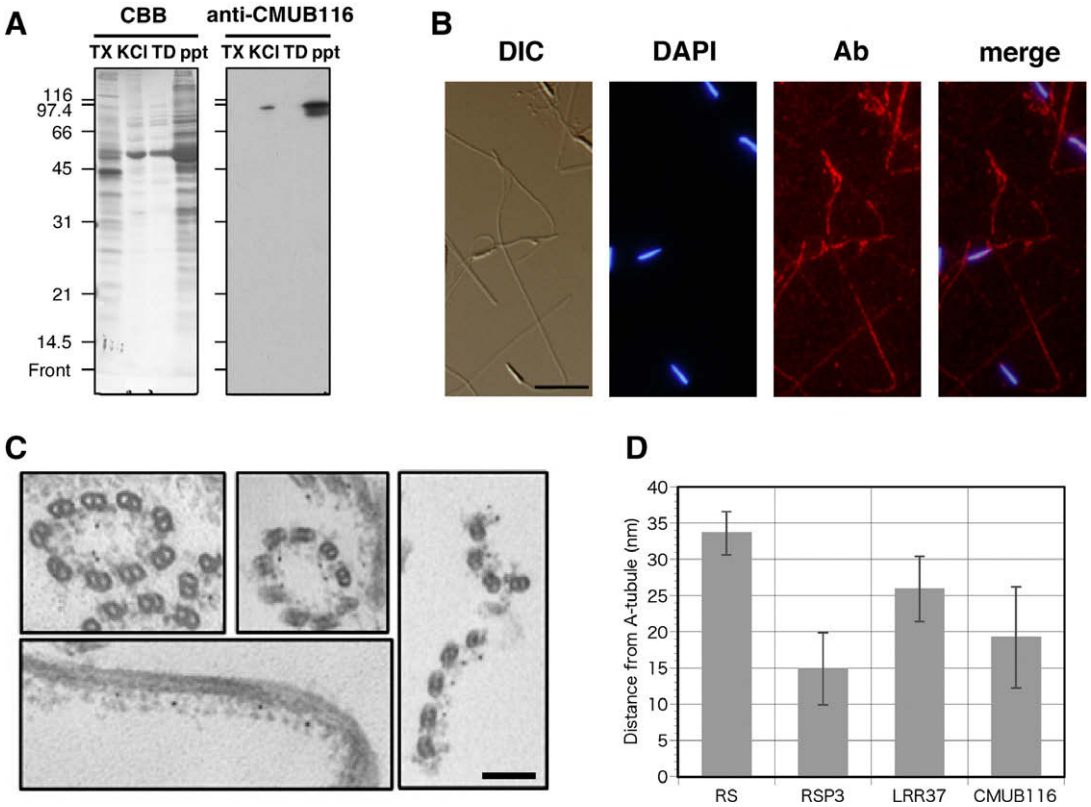

Fig. 3. Localization of CMUB116. (A) Western blot of several fractions from *Ciona* sperm axonemes. Sperm flagella were fractionated into Triton X-100 extract (TX), 0.6 M KCl extract of the axonemes (KCl), the supernatant (TD), and the pellet (P) after Tris–EDTA treatment of the KCl-extracted axonemes. Each fraction was examined by Western blotting with antibodies against several radial spoke proteins. (B) Immunofluorescence microscopy. The localization of CMUB116 along sperm flagella is clearly seen. Sperms were fixed, stained with antibody (red), and counterstained with DAPI (blue). Differential interference contrast and merged fluorescent images also are shown. Bar, 10 μm. (C) Immunogold electron microscopy. 0.6 M KCl-treated axonemes from *Ciona* sperm were labeled with antibodies as indicated, followed by labeling with 5 nm gold-conjugated anti-mouse IgG antibody. Bar, 100 nm. (D) Statistic analysis of the distance from the base of radial spoke to gold particles in immunogold images. The distance was measured on a NIH-image program. S.D. for each are indicated as a bar. The difference between the average values for Ci-RSP3 and CMUB116, or those for LRR37 and CMUB116 was shown significant by Student's *t*-test ($P < 0.001$).

In contrast to the overall sequence similarities in radial spoke proteins between *Ciona* and *Chlamydomonas*, protein domain/motif structures were not well retained in corresponding homologs. Rather, domain/motif structures were distributed in different spoke components between these organisms, like the case of NDK and Dpy-30 domains in NDK/DPY26. Another example was IQ motif: it was found in RSP23 in *Chlamydomonas*, but is found in CMUB116 in *Ciona*. Unlike RSP23, CMUB116 contains no NDK domain; however it possesses an ubiquitin domain. This feature well agrees with the idea that has been recently proposed as "module-dominant conservation" of the axonemes [24]: the molecular structure of orthologous component of radial spoke proteins became diverse but the domain/motif structures in the complex have been conserved, possibly resulting in the conservation of ultrastructure and function. This is also supported by a recent proteomic analysis of the subunits of outer arm dyneins [18].

Amongst the domains present in *Ciona* spoke proteins, the ubiquitin domain is of particular interest, as ubiquitin-like domain proteins (UDPs) are known to interact with the 26 S proteasome [25]. CMUB116 is the first axonemal protein that contains an ubiquitin domain and it is worthwhile mentioning that sperm flagellar motility is regulated by a proteasome-dependent pathway [26]. The results of a homology search showed that CMUB116 has homologs in human and mouse displaying similar domain organization and protein size. A BLASTP search against the *Chlamydomonas* genome database found a protein, FAP253, with sequence similarity to CMUB116. FAP253 contains an IQ motif but no ubiquitin domain (Fig. 2, Table 3).

We raised a specific antibody against CMUB116 and immunologically examined the characteristics of CMUB116 (Fig. 3A). Immunofluorescence microscopy showed the localization of CMUB116 along entire length of flagella (Fig. 3B). Immunogold electron microscopy clearly showed its localization on radial spoke (Fig. 3C). The distance of gold particles from the base of A-tubules of outer doublet was statistically analyzed and compared with those of RSP3 and LRR37 (Fig. 3D). RSP3 or LRR37 is known to locate at the base or head of radial spoke [20]. The full length of radial spoke was estimated as 33.6 ± 2.6 nm. The distance from the base of A-tubule to the gold particles in the case of CMUB116 (19.2 ± 6.6 nm) was significantly apart from that of Ci-RSP3 (14.9 ± 4.8 nm) or LRR37 (25.9 ± 4.3 nm). This suggests that CMUB116 is a protein localized in the stalk of radial spoke.

Extraction of axonemes followed by immunoblotting showed that CMUB116 mostly remains in the pellet. Other spoke proteins such as RSP3, MORN40, Ci-AxHsp40, Ci-RSP9 and NDK/DPY26 display a similar behavior following Tris–EDTA (TD) extraction. Partial extraction of CMUB116 and MORN40 could be achieved using buffers containing 0.6 M KCl (Fig. 4A). Further purification of this axonemal KCl extract by subsequent sucrose density gradient centrifugation and MonoQ column chromatography steps showed co-migration and co-elution of CMUB116 and MORN40, suggesting that both proteins are associated in a tight complex. A protein component of central pair apparatus Ci-Spag6, was also extracted by 0.6 M KCl solution. However, it was separated from CMUB116/MORN40 complex in both sucrose density gradient centrifugation and MonoQ column. Furthermore the colocalization of CMUB116 and MORN40 to radial spokes was demonstrated by immunogold electron microscopy (Fig. 4D). Chemical cross-linking experiment with EDC showed that a ~160 kDa cross-linked product was formed in the immunoprecipitate with anti-RSP3 antibody, suggesting that MORN40 was directly associated with a ~120 kDa component, most likely CMUB116 (Fig. 4E).

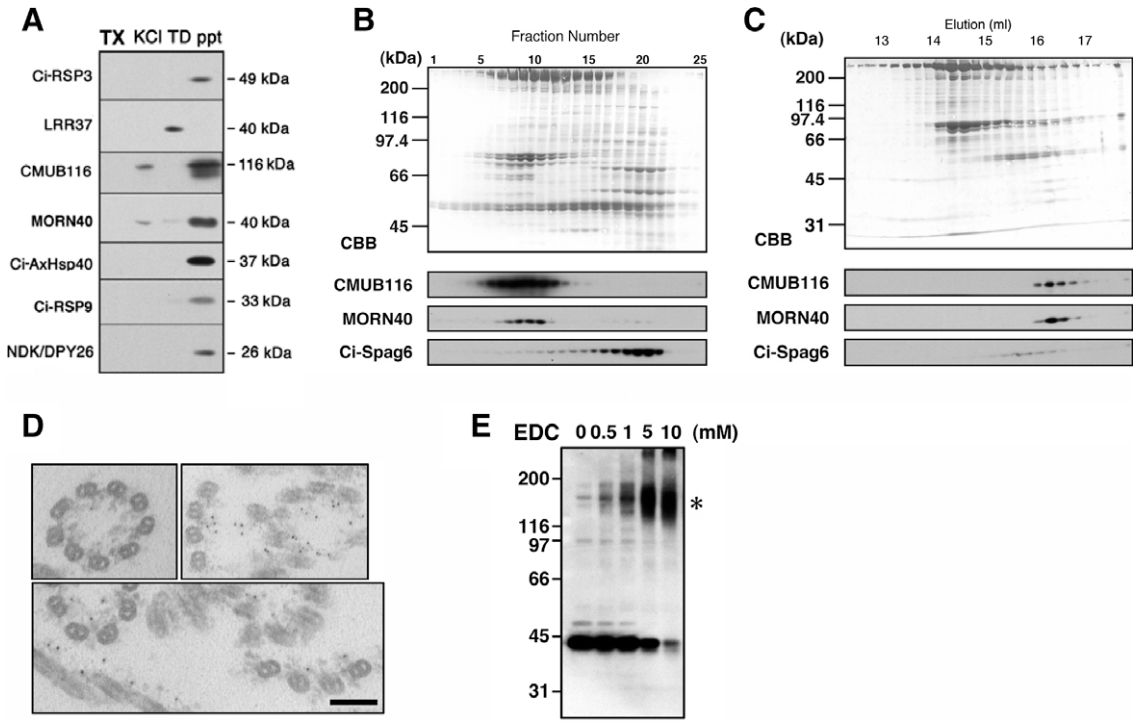

Fig. 4. Association of CMUB116 and MORN40 in axonemal KCl extract. (A) Immunoblotting of flagellar fractions with antibodies against several radial spoke proteins. A part of CMUB116 and MORN40 was extracted by 0.6 M KCl. (B) Separation of KCl extract by sucrose density centrifugation. Fractions were collected from the bottom of the gradient tube. Sedimentation profiles of CMUB116, MORN40 and Ci-Spag6, a central pair protein, were analyzed by Western blotting. CMUB116 and MORN40 comigrated through sucrose density gradient. (C) Separation pattern of the proteins in fractions 5–8 from sucrose gradient in by MonoQ anion-exchanging column chromatography. Both CMUB116 and MORN40 were eluted in the same fractions but separated from Ci-Spag6. (D) Immunogold localization of MORN40 at radial spokes. Bar, 100 nm. (E) EDC cross-linking of radial spokes isolated by immunoprecipitation with anti-RSP3 antibody. Immunoblotting with anti-MORN40 antibody detected ~160 kDa cross-linked product (asterisk).

In conclusion, we identified several radial spoke components of *Ciona* sperm axonemes. Comparison of these components with the radial spoke proteins from *Chlamydomonas* reveals both common and diverse features in radial spoke components and supports the "module-dominant conservation" of axonemes [24]. CMUB116, a protein containing an ubiquitin domain, was identified in this study. CMUB116 appears to be tightly associated with MORN40 and located at the stalk of radial spokes. The role of the ubiquitin domain, in conjugation with the potential role of an IQ motif in the regulation of radial spokes, requires further investigations. Further functional study on CMUB116 promise to reveal new insights in the regulatory mechanism of sperm radial spokes in flagellar motility.

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